Reference: *Biol. Bull.* 171: 450–460. (October, 1986)

COLD-INDUCED APOLYSIS IN ANECDYSIAL BRACHYURANS

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Abstract

Several integumentary tissues of brachyuran crabs separate from the exoskeleton (undergo apolysis) when exposed to low temperatures (0°C) for one hour or longer. Apolysis did not occur in crabs held at room temperature for the same length of time following the destruction of the subesophageal ganglia *i.e.,* the phenomenon was not due to the death of the animals. Apolysis did occur in animals chilled following ganglionectomy. Cold-induced apolysis occurred in species of seven families of brachyurans distributed throughout tropical, North Atlantic, or Pacific Northwest habitats. Therefore, it is not merely due to chilling subtropical animals. Adult majid crabs, which are in terminal anecdysis, underwent cold-induced apolysis as did Bermuda land crabs, the latter in all stages of the molt cycle tested. Chilling *in vitro* also induced apolysis in isolated pieces of integumentary tissue. The biological significance of these observations is that the mechanisms underlying apolysis may be activated in the integumentary tissues of anecdysial animals.

Introduction

One of the most definitive events during the crustacean molt cycle is the separation of the epithelium from the membranous layer (ML), the innermost layer of the exoskeleton (Skinner, 1962, 1985); this process has been termed apolysis (Jenkin and Hinton, 1966). Apolysis is one of the earliest responses to increases in titers of the molting hormone, 20-OH ecdysone, that occur in proecdysis (see Skinner, 1985, for references). In the normal molt cycle, apolysis signals the initiation of some of the major metabolic and physiological processes that occur prior to ecdysis. In large specimens of Bermuda land crabs, *Gecarcinus lateralis,* apolysis occurs approximately one month before ecdysis following which the ML and ~75% of the endocuticle are degraded and the epicuticular and exocuticular layers of the new exoskeleton are formed (Skinner, 1962).

We describe here a phenomenon we call cold-induced apolysis. Specifically, we have observed that the epithelium and underlying muscle from chelae as well as the integumentary tissues of branchiostegite regions can be detached easily and cleanly from the exoskeleton if animals are chilled on ice for 0.5 to 4.5 h. The phenomenon also occurs in autotomized chelae and excised pieces of branchiostegites similarly treated. Cold-induced apolysis was observed in members of seven families of brachyurans, including tropical land crabs as well as a number of species of marine crabs that commonly experience temperatures of 10°C and less; it was not observed in

Received 25 February 1986; accepted 22 July 1986.

Research sponsored by grants from the National Science Foundation and the Exploratory Studies Program of the Oak Ridge National Laboratory and by the Office of Health and Environmental Research, U. S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

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specimens from the two astacuran families that were examined. These observations imply that the mechanisms underlying at least some aspects of apolysis are present in the integumentary tissues of anecdysial animals. Only a trigger is needed for their release.

Cold-induced apolysis facilitates the recovery of intact crustacean soft tissues and provides a useful method in the analysis of such tissues. Brief reports of some of these results have been published (O'Brien *et al.*, 1984; O'Brien and Skinner, 1985).

Materials and Methods

Animals

Specimens of *G. lateralis* were from Bermuda and *Rhithropanopeus harrisii* specimens were from the Chesapeake Bay, Maryland, or near the Duke Marine Laboratory, Beaufort, North Carolina. *Pugettia producta, Taliepus nuttallii, Cancer antennarius,* and *Pachygrapsus crassipes* were collected from Santa Barbara, California. *Gecarcinus ruricola, Cardisoma guanhumi,* and *Menippe mercenaria* were taken from Fort Pierce, Florida. *Callinectes sapidus, Ocypode quadrata, Uca pugilator,* and *Sesarma cinereum* were collected near the Duke Marine Laboratory. Crayfish [*Cambarus bartonii bartonii* (Cambaridae)] were collected locally and lobsters [*Homarus americanus* (Nephropidae)] were purchased from a local market.

Treatment of animals

Assays on *G. lateralis* and *R. harrisii* were performed in Oak Ridge where these two species were maintained. All other assays were performed near the collecting sites.

Experimental animals were packed in shaved ice. The subesophageal ganglia of some control animals were destroyed with a dissecting needle and the animals were placed in a humidified environment at 23°C or chilled in ice until they were dissected. We scored as positive a clean and complete separation of (i) the carapace from the muscles of the cardiac stomach when the carapace was lifted from the posterior border or (ii) the exoskeleton from the underlying integumentary tissues or muscle.

Histology and electron microscopy

Tissues were removed from stage-C_4 *G. lateralis* for examination by light and electron miscroscopy. In the *in vivo* treatments, whole animals were chilled before branchiostegite segments were removed for fixation. Segments of branchiostegites including calcified exoskeleton, ML, and integument were taken with a small rotary power saw. Segments of branchiostegites were removed from five animals for studying *in vitro* cold-induced apolysis; they were placed in sealed plastic bags or plastic wrap before being packed in ice. Following treatment, the ML was peeled from the calcified exoskeleton before fixation. For light microscopy, samples were fixed in Bouin's solution, cut to a thickness of 5 μm at 50 μm intervals, and stained with hematoxylin-eosin. For electron microscopy, samples were fixed 2 h in 2.8% ultrapure glutaraldehyde, 0.57 *M* glucose, and 0.10 *M* sodium cacodylate, pH 7.4, and postfixed 2 h in 2% osmium tetroxide, 0.57 *M* sucrose, and 0.10 *M* sodium cacodylate, pH 7.4 (Mykles and Skinner, 1981). Sections were stained with uranyl acetate and lead citrate and examined with a Philips 400T electron microscope operated at 80 kV.

FIGURE 1. Muscles (bottom) removed from propoda and dactyls of autotomized chelae of two *G. lateralis* following 4 h at 0°C. Muscles have also separated from the apodemes which are visible inside the exoskeletal "gloves" (top).

RESULTS

Cold-induced apolysis of the epithelium of the chelae in *G. lateralis* required 3 to 4 h (Fig. 1). Separation of the integumentary tissues occurred in both intact whole animals (Fig. 2A; Table I) and those dying following ganglionectomy when chilled at 0°C (data not shown). Integumentary tissues also separated from the ML when they had been removed from crabs and chilled *in vitro* (Fig. 2B). Large segments of epithelium and groups of tendinal cells (Koulish, 1973) appeared intact after separation. The amount of epithelial tissue adhering to the ML varied considerably even within one individual and following treatment either *in vivo* or *in vitro*. There was little or no cellular debris attached to some areas of the ML (Fig. 3A) while other areas had epithelial cells and parts of tendinal cells present (Fig. 3B, C). Cellular debris remained with ~50% of the microscopic fields of the ML of *in vitro* preparations of branchiostegite regions. In unchilled branchiostegite segments taken from specimens of *G. lateralis* before exposure to 0°C (Fig. 4A) and from dying (ganglionectomized) animals and kept at room temperature (Fig. 4B), the epithelial and tendinal cells of the integument remained attached to the ML. In summary, separation always occurred in chilled tissue and only in chilled tissue.

In electron micrographs of segments of ML from animals induced to undergo apolysis *in vivo*, integumentary tissues had separated completely. Cells appeared to be intact and there was no evidence of lysis. A flocculent material similar to that found in hemolymph sinuses (Fig. 5A) was present in the space between the ML and integumentary tissue; it may have been hemocyanin that had precipitated during fixation. The epithelium of untreated animals (Fig. 5B) as well as of dying ganglionectomized animals kept at room temperature was firmly attached to the ML. Tendinal cells were filled with microtubules whose orientation was perpendicular to the plane of the epithelium and cuticle (Fig. 5C). These microtubules formed junctions with tonofibrillae of the ML in tissues not exposed to 0°C. Although microtubules in mammalian cells can dissociate at 0°C (Kirschner, 1978), electron micrographs showed

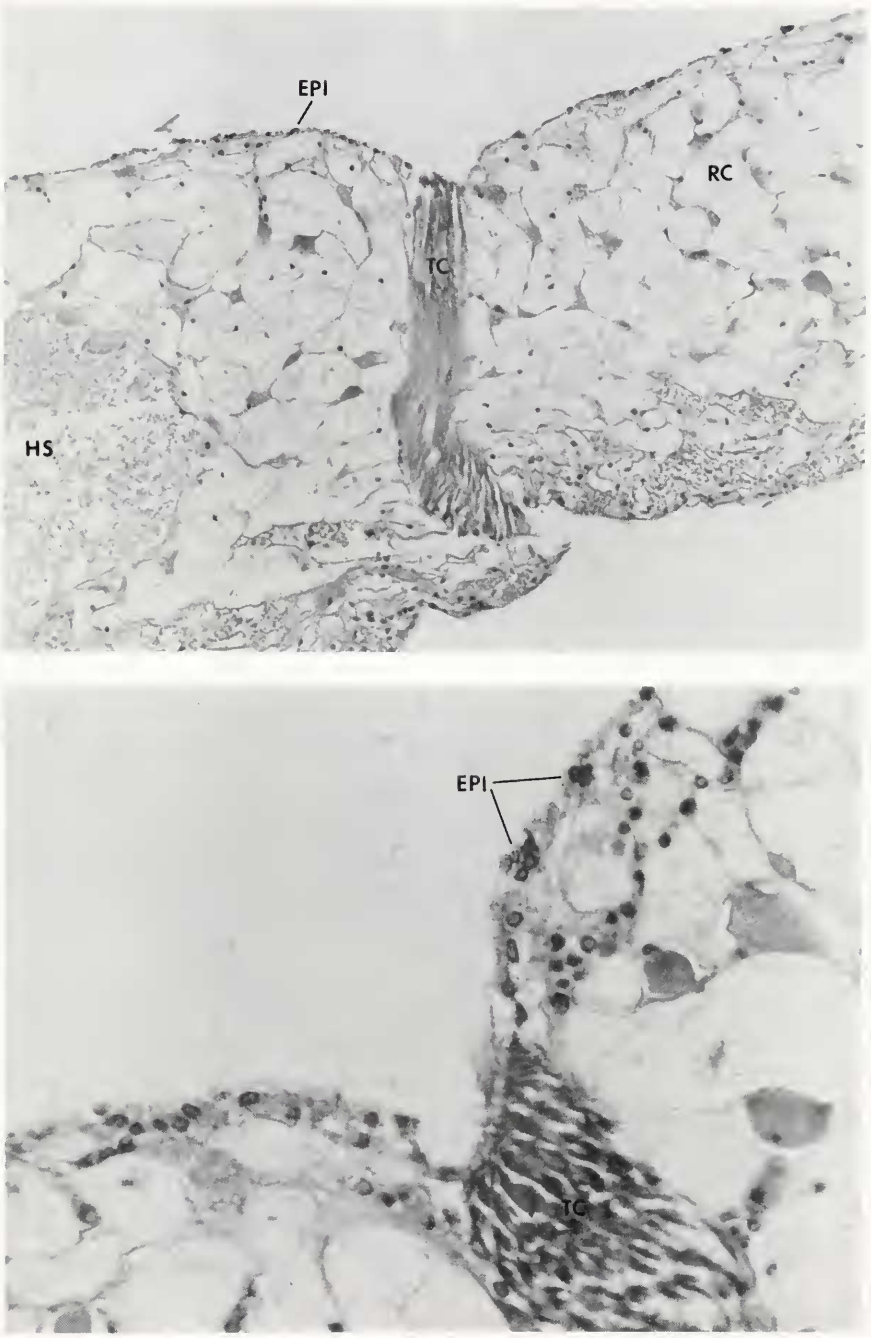

FIGURE 2. Transverse sections of integumentary tissues from branchiostegites that did undergo cold-induced apolysis. (A) *G. lateralis* chilled 1.5 h *in vivo*, then tissues removed, ×167. (B) Tissues removed from *G. lateralis*, then chilled 1.5 h *in vitro*, ×425. EPI, epithelial tissue; HS, hemolymph sinus; TC, tendinal cells; RC, reserve cells.

TABLE I

Brachyurans in which cold-induced apolysis occurred

Family	Species
Cancridae	*Cancer antennarius*
Gecarcinidae	*Cardisoma guanhumi*
	Gecarcinus lateralis
	Gecarcinus ruricola
Grapsidae	*Pachygrapsus crassipes*
	Sesarma cinerum
Majidae	*Pugettia producta*
	Taliepus nuttallii
Ocypodidae	*Ocypode quadrata*
	Uca pugilator
Portunidae	*Callinectes sapidus*
Xanthidae	*Menippe mercenaria*
	Rhithropanopeus harrisii

that the microtubules in the tendinal cells following cold-induced apolysis were not in disarray (data not shown).

Cold-induced apolysis was observed in species of all seven brachyuran families tested (Table I). Although the integumentary tissues of most specimens separated from the carapace within one h, the response for *R. harrisii* required 4.5 h. If animals were submerged in the ice-water at the bottom of the container, cold-induced apolysis did not occur. In adult majid crabs, *P. producta,* complete separation of the integumentary tissues from the exoskeleton at the cardiac region of the carapace required about 30 min exposure to 0°C and at the branchiostegite region of the carapace about 45 min (Table II). None of the controls, including ganglionectomized crabs kept at room temperature for 3 h, underwent apolysis. The integumentary tissues of crayfish branchiostegites remained firmly attached to the ML and the assays were scored as negative. All crayfish were alive following 3.5 h in ice. Although lobster integumentary tissues remained attached to the ML after 4 h of chilling and the assays were scored as negative, one of us found that chilling lobster claws led to partial separation and facilitated the removal of muscle (Mykles, 1985; Mykles and Skinner, 1986).

Cold-induced apolysis occurred in *G. lateralis* in early proecdysis (D_0, Skinner, 1962) metecdysis, and anedcysis. No animals in stage A of metecdysis were tested, but in stage B, which occurs well before the formation of the membranous layer (Drach, 1939; Skinner, 1962), the integumentary tissue of the branchiostegites could be easily separated from the exoskeleton following exposure to 0°C. Cold-induced apolysis was also observed in adult *P. producta* and *T. nuttallii,* which were in terminal anecdysis (Tessier, 1935; Hartnoll, 1963; O'Brien, 1984).

DISCUSSION

Results were equivocal as to whether the plane of separation during cold-induced apolysis occurred between the epithelium and the superficial ML or between the epithelium and the underlying tissues of the integument. Some segments of ML from animals that had undergone cold-induced apolysis had no tissues attached (Fig. 3A, 5A), while other segments had epithelial cells and fibers from tendinal cells present (Figs. 3B, C). The same degradative enzymes may be active during physiological and cold-induced apolysis despite the presence of fragments of epithelial and/or tendinal

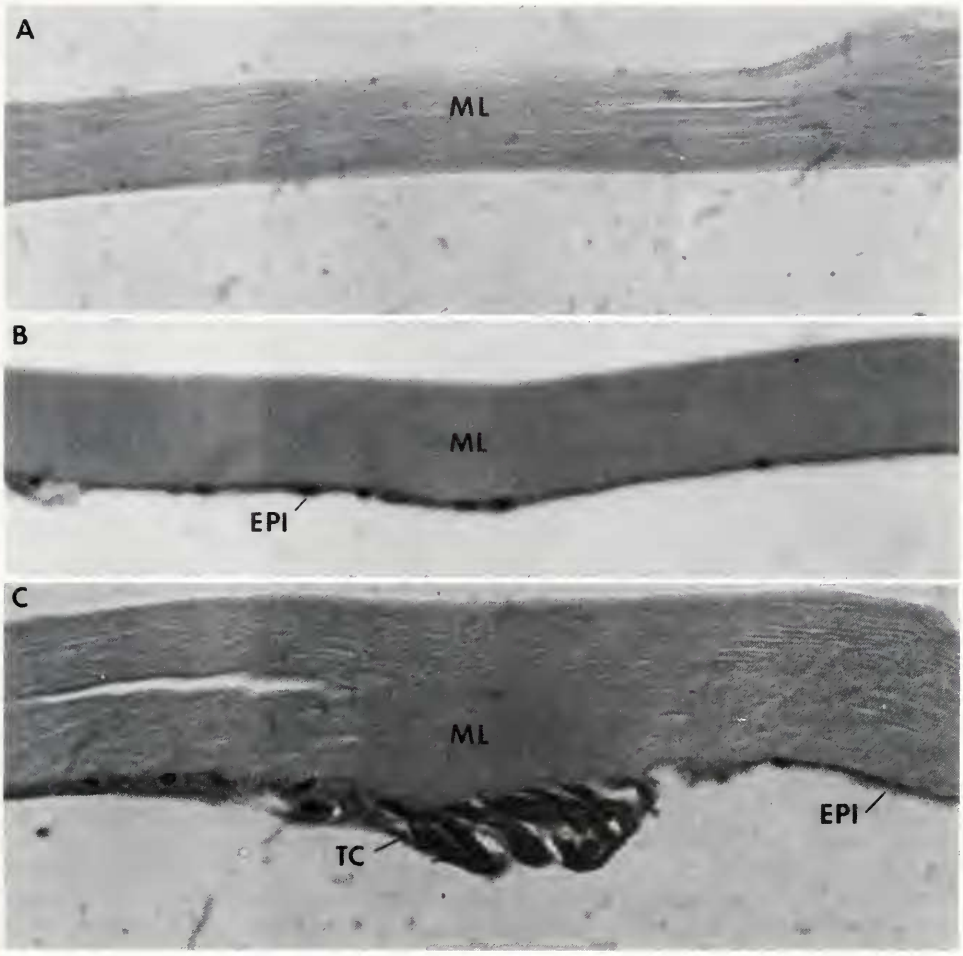

FIGURE 3. Transverse sections of ML from *G. lateralis* in (Fig. 1B) that did undergo cold-induced apolysis. Branchiostegites had been chilled for 1.5 h after removal from animal. Integumentary tissues and ML were then detached from each other and from the remainder of the exoskeleton, ×310. (A), (B), (C) Three areas of ML. Abbreviations as in Figure 2.

cells on some areas of ML following cold-induced apolysis; perhaps the incubation period was too short for complete separation to ensue.

Cold-induced apolysis occurred in tissues that had been isolated from crabs before exposure to 0°C (Fig. 2B, 3A–C) indicating that it is not a neural response. Electron micrographs of crab integumentary tissues indicated that cold shock did not cause the depolymerization of microtubules that can occur at 0°C in mammalian cells (Kirschner, 1978). Cold shock may have stimulated release into the apical extracellular environment of the integumentary tissues degradative enzymes responsible for apolysis. Such "apolysases" could have been stored within vesicles of epithelial cells (Koulish and Klepal, 1981) or in the several types of glands found in crustacean integumentary tissues (Skinner, 1962; Babu *et al.*, 1985). It is unlikely that cold-induced apolysis is due to lysosomal breakdown following cell death since it occurred within 1 h at 0°C but was not observed in dying animals even after 3 h at room

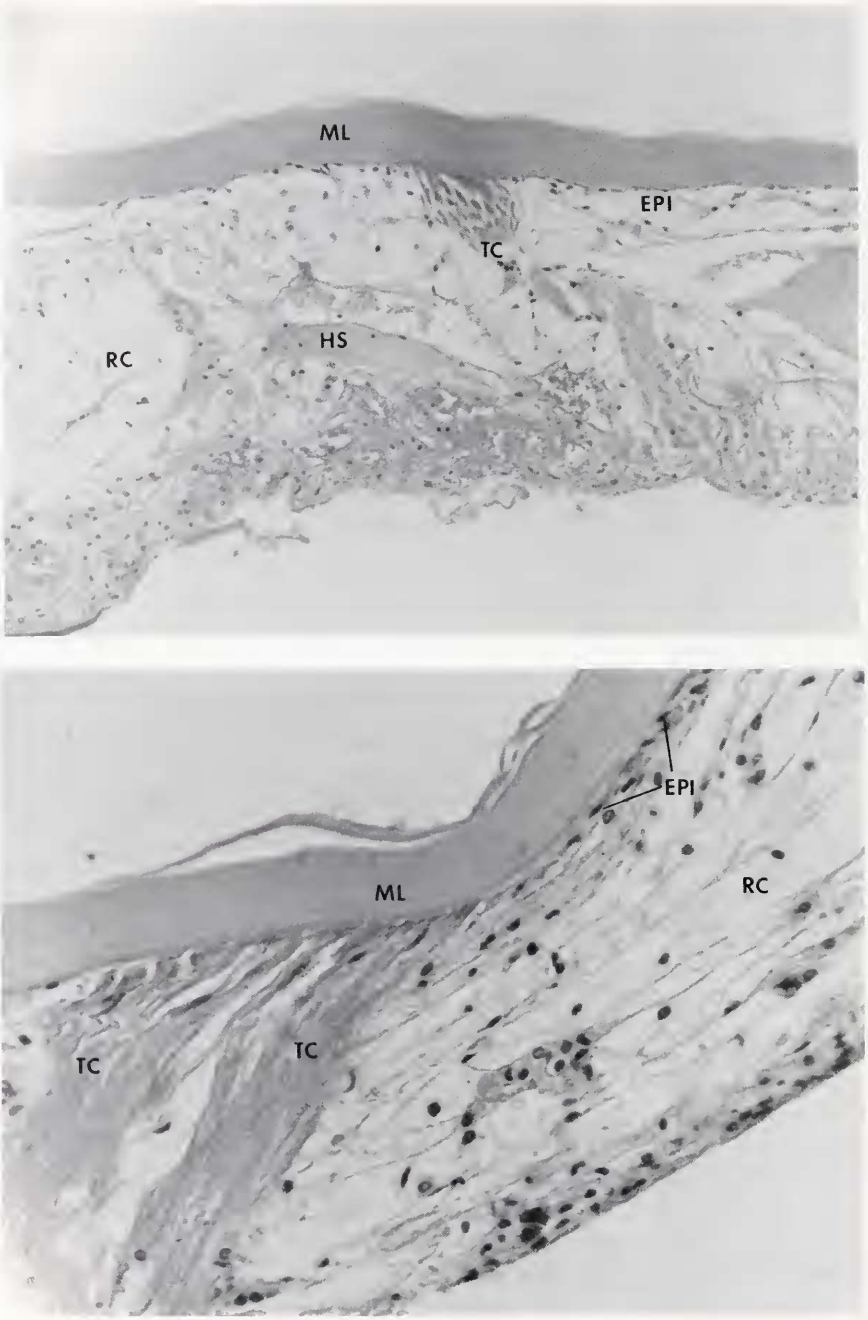

FIGURE 4. Transverse sections of integumentary tissues from branchiostegites from two *G. lateralis* that did not undergo cold-induced apolysis. (A) Untreated animal, ×167. (B) Tissues from ganglionectomized crab held at room temperature for 1.5 h, ×425. Abbreviations as in Figure 2.

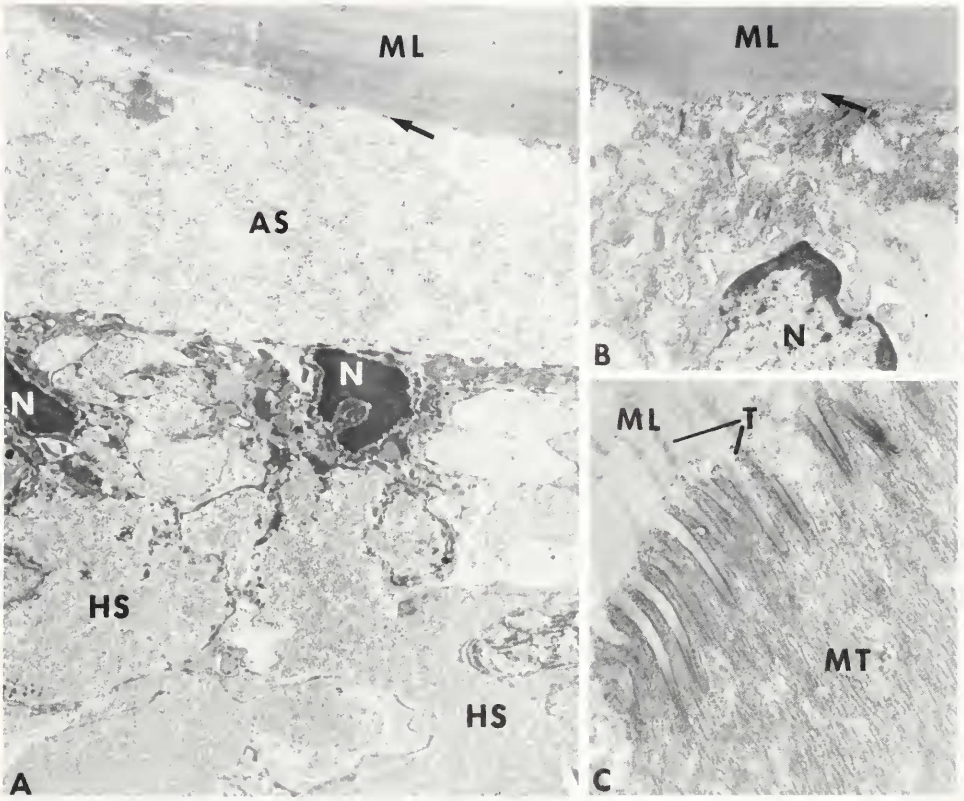

FIGURE 5. Electron micrographs of transverse sections of branchiostegite segments from two *G. lateralis*. (A) Crab chilled and then tissue removed. Epithelium completely separated from ML. No cellular material adhered to inner surface (arrow) of membranous layer, ×6700. (B and C) Tissues removed from untreated animal. (B) Epithelial cell attached (arrow) to ML, ×9600. (C) Junctions between microtubules (MT) of tendinal cell and tonofibrillae (T) of ML, ×20,000. AS, apolytic space; N, nuclei of epithelial cells; other abbreviations as in Figure 2.

temperature. It is also unlikely that cold-induced apolysis is caused by the release of cytoplasmic proteinases following rupture of cell membranes; freezing of cytoplasm occurs well below 0°C. Furthermore, cold-induced apolysis appeared to be localized near if not at the epithelial-cuticle interface with minimal disruption of underlying tissues removed from whole animals that had been chilled (Figs. 2A, 5A). After chilling, muscle fibers generated membrane potentials of −50 to −60 mv (unpub. obs.) suggesting that the surface membranes were intact. The induction of separation between the amuscular integumentary tissues and exoskeleton of the branchiostegites showed that cold-induced apolysis was not due to a reflexive contraction of muscle.

Localized differences in the length of time required to cause cold-induced apolysis may reflect the distribution and abundance of tendinal cells, which function to anchor muscle fibers and integumentary tissues to the exoskeleton. Tendinal cells are attached to tonofibrillae within the exoskeleton via specialized junctions (Green and Neff, 1972; Koulish and Klepal, 1981). At sites where muscles insert into the cuticle, such as the chelae, large numbers of such attachments would be detached as cold-induced apolysis occurred. The cardiac and branchiostegite regions, with many fewer muscle insertions, require less time to undergo cold-induced apolysis. Differences in

TABLE II

Length of time specimens of Pugettia producta *were chilled before separation of the MLB from underlying tissues*

Time (min)	Separation of membranous layer from			
	Stomach muscle		Branchiostegite integument	
	0°C	23°C	0°C	23°C
0	−	−	−	−
10	−	−	−	−
30	+	−	−	−
45	+	−	+	−
60	+	−	+	−

+ indicates separation occurred; − indicates separation did not occur.

the distribution of tendinal cells may also explain why apolysis does not occur uniformly over the entire surface of the proecdysial animal (Drach, 1939; Koulish and Klepel, 1981).

Degradation of the crustacean exoskeleton during proecdysis involves the secretion of chitinases, chitobiases, and proteinases by the underlying integumentary tissues and is thought to be regulated by the molting hormone 20-OH ecdysone. Whether any of these enzymes are activated or newly synthesized in response to chilling is not known. Activity of crustacean degradative enzymes has been observed at 0°C; extensive lipolysis in three species of krill either maintained at 0°C or frozen (Saether *et al.,* 1986) was attributed to post mortem proteolysis (Saether *et al.,*1986 citing the Ph.D. thesis of Ellingsen, 1982). Since dying (ganglionectomized) *G. lateralis* did not undergo apolysis when kept at room temperature for 3 h, cell lysis cannot account for the phenomenon we describe.

Many crustaceans exhibit indeterminate growth, molting throughout their lives (Hartnoll, 1982). For example, even very large specimens of *G. lateralis* enter proecdysis following autotomy of a critical number of limbs (Skinner and Graham, 1970; Holland and Skinner, 1976). We observed that metecdysial and anecdysial *G. lateralis* did undergo cold-induced apolysis. The simplest interpretation, assuming that the same degradative enzymes are involved in both proecdysial apolysis and cold-induced apolysis, would be that the relevant enzymes are synthesized during metecdysis. However, the ability of crabs that exhibit determinate growth to undergo cold-induced apolysis requires a modification of this explanation. With the exception of males of the genus *Chionoecetes* (Watson, 1970), adult crabs in the family Majidae (Tessier, 1935; Hartnoll, 1963; O'Brien, 1984) are like adult *Leuroleberis zealandica* (Ostracoda) (Fenwick, 1984) and adult copepods (Hartnoll, 1982); they do not molt again. Cold-induced apolysis in adult majids suggests that at least some of the enzymes involved in apolysis may not be synthesized during or immediately prior to the proecdysial period. They may be constitutive in the integumentary tissues of some brachyurans. This could also account for the presence of chitinase activity in integumentary tissues of crabs in all stages of the molt cycle (Jeuniaux, 1959).

We suggest that mechanisms similar to those responsible for cold-induced apolysis may cause the separation of tissues from the exoskeleton of crabs bitten by an octopus. Although crab prey were separated from *Octopus* predators immediately after paralysis was induced by a single bite, the tissues of the crabs could be separated

intact from the exoskeleton within 30 min (Nixon, 1984). Nixon postulated that the octopus injected enzymes highly specific for the arthrodial membrane and musculoskeletal attachments since, in another experiment, crab tissues were recognizable 2 h after ingestion by the octopus. Our data suggest that instead of exogenous proteinases, the octopus may inject substances that stimulated crab integumentary tissues to synthesize, activate, or secrete degradative enzymes.

Cold-induced apolysis might influence conclusions drawn from some growth studies. The growth rate of populations of crustaceans is sometimes estimated from the percentages of animals in proecdysis and metecdysis (Hartnoll, 1982). Because apolysis is such an easily recognizable characteristic, any crustacean with an exoskeleton separated from the underlying tissues is likely to be classified as proecdysial without further inspection. Our data indicate that, at least for some Brachyura, if individuals in the metecdysial and anecdysial stages are kept on ice for extended periods of time before examination, they may be incorrectly judged to be in proecdysis.

The inability of the two astacurans to undergo cold-induced apolysis is not surprising given their exposure to 0°C and below in their natural habitats (Squires, 1970; Becker *et al.*, 1977; Mirenda and Dimock, 1985). *R. harrisii*, an estuarine crab, is also exposed to low temperatures during the winter. Turoboyski (1973) reported that a European subspecies of *R. harrisii* survived exposure to ice for short periods, in agreement with our observation that cold-induced apolysis in *R. harrisii* required a longer exposure to ice than the other brachyurans tested. The occurrence of apolysis after chilling in *R. harrisii* emphasizes the fact that the phenomenon is not limited to tropical or even subtropical crabs. We observed cold-induced apolysis in a number of temperate species that tolerate low temperatures (Table I). *Callinectes sapidus* is commonly found at Cape Cod and in the North Sea (Williams, 1984) while the ranges of *Cancer antennarius* and *Pachygrapsus crassipes* extend northward to Oregon (Morris *et al.*, 1980). *Pugettia producta*, which underwent cold-induced apolysis in only 45 min (Table II), is found at even higher latitudes in the cold waters off British Columbia and Alaska (Morris *et al.*, 1980).

The mechanism that causes cold-induced apolysis may have been modified by selective pressure in crustaceans that tolerate 0°C. This possibility is strengthened by preliminary results that demonstrate the presence of alkaline cysteine proteinase activity in extracts of integumentary tissues of *G. lateralis* but not in those of lobster (O'Brien and Skinner, 1985), a species that did not undergo cold-induced apolysis.

ACKNOWLEDGMENTS

We thank the Bermuda Biological Station for providing *G. lateralis*, Drs. A. Wenner, J. Costlow, and T. Bailey for temporary laboratory space, T. Soper for photography, M. Morita for use of the electron microscope, W. Berger for determining the resting potentials in muscle, Mr. J. Wesley for histological services and Ms. Nette Crowe for help with preparation of the manuscript. *R. harrisii* were supplied by Dr. A. Hines and *P. crassipes* by Y. Ricard. G. Thorpe and J. O'Brien IV assisted in collecting *C. sapidus* and crayfish, respectively; the latter were identified by D. Cox.

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